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DOI: https://doi.org/10.1016/j.tibs.2020.11.002

Trends in
Biochemical Sciences

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Review

The Hammer and the Dance of Cell Cycle Control

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Cell cycle checkpoints secure ordered progression from one cell cycle phase to the next. They are important to signal cell stress and DNA lesions and to stop cell cycle progression when severe problems occur. Recent work suggests, however, that the cell cycle control machinery responds in more subtle and sophisticated ways when cells are faced with naturally occurring challenges, such as replication impediments associated with endogenous replication stress. Instead of following a stop and go approach, cells use fine-tuned deceleration and brake release mechanisms under the control of ataxia telangiectasia and Rad3-related protein kinase (ATR) and checkpoint kinase 1 (CHK1) to more flexibly adapt their cell cycle program to changing conditions. We highlight emerging examples of such intrinsic cell cycle checkpoint regulation and discuss their physiological and clinical relevance.

From Stop and Go Cell Cycle Decisions to a Continuum of Deceleration and Brake Release Mechanisms

Cell cycle checkpoints (see Glossary) are control mechanisms that ensure proper and ordered progression of cells through the cell cycle [1]. Genome integrity maintenance is among the key tasks of cell cycle checkpoint control, and DNA damage checkpoints have thus evolved to trigger cell cycle arrest in response to genomic lesions and allow time for repair [2]. The cell cycle checkpoint model, in agreement with the general meaning of the term checkpoint, implies certain criteria, which have to be met by a cell to continue its progression through the cell cycle (the 'go' decision) and which, if they are not fulfilled, lead to transient or permanent cell cycle arrest (the 'stop' decision). Among the major cell cycle checkpoints are the G1/S checkpoint, the G2/M checkpoint, and the spindle checkpoint in mitosis. By analogy to 'the hammer and the dance' metaphor introduced early in 2020 by Tomas Pueyo to emphasize the importance of acting quickly and forcefully to contain the global health threat posed by SARS-CoV-2 and later ease and adapt the measures to continually manage and control COVID-19 ('Coronavirus: the hammer and the dance', published online March 19, 2020; https://medium.com/@tomaspueyo/coronavirus-the-hammer-and-the-dance-be9337092b56), we can consider full checkpoint activation to halt cell cycle progression as 'the hammer', which can be an effective means to minimize further damage in the face of severe threats to genome integrity. However, rather than relying exclusively on binary stop and go decisions, a picture is starting to emerge in which the cell cycle control machinery uses fine-tuned brakes and brake release mechanisms [3], thereby temporarily decelerating certain processes while continuing others, to globally balance genome surveillance with cell cycle progression ('the dance'). Thus, cell cycle arrest as an extreme measure seems reserved for severe events that threaten genome integrity and cell survival, while more moderate and adaptable measures are commonly used by cells to deal with controllable genomic lesions and replication intermediates associated with endogenous replication stress in a more flexible and context-specific manner.

In this review, we aim to provide a synthesis of recent findings that shed new light on cell cycle checkpoint functions during unperturbed cell proliferation and in the presence of endogenous

Highlights

Shutdown of cell cycle progression by checkpoint activation is the exception rather than the norm when cells face physiological levels of replication stress.

In response to endogenous replication stress, cells use tunable deceleration and brake release mechanisms under the control of the ATR and CHK1 kinases for timely completion of DNA duplication.

Intrinsic checkpoint activation, maintenance, and recovery represent a continuum, which is modulated by CHK1 phosphorylation and ubiquitin-dependent proteasomal degradation.

Sharp cell cycle transitions, in which one cell cycle phase is fully concluded before the next one begins, can be blurred due to a balancing act between genome integrity maintenance and an urge for cell cycle completion.

Deviations from ordered cell cycle phase transitions promote cancer, with therapeutic opportunities for cell cycle checkpoint kinase inhibitors.

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and mild forms of exogenous replication stress, with a particular focus on **replication checkpoint** signaling in mammalian cells. We discuss sources of endogenous replication stress (i.e., replication challenges occurring during normal cell cycle progression and, in an exacerbated manner, during cellular transformation and cancer development). Rather than providing an elaborate characterization of the many specialized factors that are involved in resolving these problems, which have been summarized more comprehensively elsewhere [4–6], we discuss how the replication program itself is buffered against such naturally occurring challenges and how replication **checkpoint maintenance** enables S-phase completion while at the same time allowing cells to resolve naturally occurring replication impediments.

We then focus on the major replication checkpoint kinases ATR and CHK1 and their functions during unperturbed replication and in response to endogenous replication stress. We introduce the emerging notion that not only the type and severity of a replication problem determine checkpoint decisions, but that the cellular response is also determined by how long a replication problem persists. Along these lines, we elucidate how low levels of endogenous replication stress are important to maintain intrinsic checkpoint functions and are thus beneficial for genome integrity, while persistent high levels of replication stress can lead to **checkpoint adaptation** and cell cycle transitions with unresolved and potentially harmful genomic lesions.

Finally, we put the concept of tunable cell cycle checkpoint control into context with currently ongoing attempts to target cell cycle checkpoint kinases therapeutically. The new insights into the regulation and the function of ATR and CHK1 during normal cell cycle progression and to deal with naturally occurring endogenous replication stress (as it can be found in many cancer cells) have direct implications for the targeted use of small molecule inhibitors against checkpoint kinases in cancer therapy. We discuss these implications and provide an outlook on how the changing cell cycle paradigm from stop and go checkpoint control to fine-tuned brake and brake release regulation may help to better understand and treat human disease.

Cell Cycle Control in the Face of Genome Duplication under Stress

Genome integrity maintenance is of great importance for cell survival and homeostasis. To preserve genome integrity and coordinate genome maintenance functions with cell cycle progression, cells use an elaborate network of intertwined surveillance pathways that coordinate cell cycle checkpoint control. Due to their important genome maintenance functions, deregulation of these signaling pathways contributes to cellular transformation, cancer, and aging. When cells experience DNA lesions, they activate the **DNA damage response (DDR)**, which is critical for sensing, signaling, and repairing the damage [7,8]. The DDR comprises a large network of proteins, which recognize different types of DNA lesions and coordinate their efficient repair. As DDR functions involve the activation of DNA damage checkpoints to slow or stop cell cycle progression, timely termination of the DDR is important for cells to resume cell cycle progression once the damage is repaired. Many lesions, which challenge genome integrity, occur during DNA replication in the form of replication intermediates and byproducts of physiological DNA transactions. As such, they blur conventional definitions of DNA damage and have started to redefine cell cycle checkpoints from binary stop and go switches towards dynamic brakes and brake release mechanisms.

Sources of Replication Stress

The sources of replication stress are manifold and can hinder replication fork progression either locally or genome wide, with implications for genome integrity maintenance and associated pathologies [9,10]. The availability of building blocks for DNA synthesis is critical for replication fork progression, and nucleotide shortage thus impairs fork speed and can lead to uncoupling

Glossary

Ataxia telangiectasia and Rad3-related protein kinase (ATR): the apical kinase responsible for the cellular response to replication stress, essential for cell proliferation and survival.
Cell cycle checkpoints: cellular control mechanisms, which ensure ordered progression through the cell cycle. Proper cell cycle checkpoint control is required to maintain genome integrity and prevent cellular transformation.
Checkpoint adaptation: the process by which cells can resume cell cycle progression despite the presence of sustained, unresolved DNA damage.
Checkpoint kinase 1 (CHK1): the main downstream effector kinase of ATR; controls cell cycle progression, origin firing, and the stabilization of stalled replication forks.
Checkpoint maintenance: the processes by which checkpoint functions remain active during normal cell proliferation to ensure ordered cell cycle progression and safeguard genome integrity maintenance.
Checkpoint termination: the process by which checkpoint signaling is switched off after successful repair of DNA damage to allow the resumption of cell cycle progression.
Chromosomal fragile sites: genomic loci, which are inherently prone to breakage (e.g., after exposure to replication stress).
Cyclin-dependent kinase (CDK) activity: CDKs are serine-threonine protein kinases, which are activated by cyclins in a cell cycle-dependent manner. CDK activity promotes cell cycle transitions and drives cell cycle progression.
DNA damage response (DDR): the cellular mechanisms responsible for sensing DNA damage, signaling its presence to the cell cycle control machinery, and repairing the genome.
Dormant origins: licensed origins of replication, which normally do not fire during DNA replication. Dormant origins serve as an important backup and are used to rescue replication where replication forks stall irreversibly.
Gene gating: the process by which actively transcribed genes are transiently positioned close to nuclear pores for the productive coupling of transcription and mRNA processing to mRNA export.
Intra-S-phase checkpoint: cell cycle checkpoint mechanisms to slow the

and exposure of single-stranded DNA (ssDNA) (Figure 1A). The rate-limiting step for the generation of deoxyribonucleotide triphosphates (dNTPs) is the conversion of nucleotide diphosphates (NDPs) into deoxyribonucleotide diphosphates (dNDPs) by the enzyme ribonucleotide reductase (RNR), and deregulation of RNR activity causes replication stress and genome instability [10]. Hydroxyurea (HU) is frequently used for RNR inhibition, but also oncogene activation can affect dNTP synthesis and unbalance dNTP pools, thereby causing replication stress and promoting mutagenesis [11]. To adapt to alterations in dNTP levels, cancer cells may sense an ensuing change in redox potential and elevated reactive oxygen species (ROS) and actively slow replication forks to prevent excessive damage [12].

Besides dNTP availability, replication fidelity is inherently linked to the DNA sequence. Repetitive sequences, which comprise large portions of the human genome, are potent sources of replication stress and genome instability (Figure 1B). They can misalign during DNA synthesis and are typically associated with more compact and thus more difficult to replicate chromatin. Repeat expansions cause several neurological and muscular diseases, and repetitive DNA sequences such as AT-rich regions are associated with **chromosomal fragile sites**, breakpoints in the genome that contribute to genomic rearrangements and mutations in cancer [13,14]. Common fragile sites (CFSs) are typically found in large transcribed domains with a low density of **replication origins** (Figure 1C), which makes them particularly vulnerable to breakage [15,16]. Repetitive sequences and inverted repeats are also prone to adopt secondary structures such as hairpins, cruciforms, and G-quadruplexes in GC-rich areas (Figure 1D). These non-B DNA structures can stall replication forks, cause DNA lesions, and are associated with translocation breakpoints in cancer [17].

Secondary structures have regulatory roles in transcription and transcription itself is an important source of endogenous replication stress. Transcription–replication conflicts can arise from direct collisions between the transcription and replication machineries (Figure 1E), which can occur in both head-on and co-directional orientation. Such conflicts evoke the formation of co-transcriptional R-loops (Figure 1F), structures that form when nascent RNAs hybridize back to the template DNA and generate an RNA–DNA hybrid while displacing single-stranded non-template DNA. Although R-loops have important physiological functions, they can also interfere with replication fork progression and lead to genome instability (e.g., under conditions of oncogene-induced replication stress) [18–20]. In particular, head-on collisions between the **replisome** and the transcription machinery promote genome-destabilizing R-loops [21,22]. Interestingly, the ssDNA-binding protein RPA, an essential constitutive component of replication forks, was recently shown to sense and suppress R-loops [23]. Furthermore, the replication and repair factors BRCA1 and BRCA2 alleviate transcription–replication conflicts and play active roles in R-loop regulation, and confer resistance to G-quadruplex-stabilizing compounds [24–26].

Replication fork progression can also be impaired by naturally occurring DNA lesions including bulky DNA adducts and by covalent DNA crosslinks (Figure 1G) [9] as well as by DNA–protein crosslinks (DPCs) ahead of the fork (Figure 1H) [27,28]. DPCs occur by linking nucleic acid-modifying enzymes to DNA as trapped reaction intermediates and by nonenzymatic crosslinking of chromatin-associated proteins, and they are emerging as an important source of genome instability [27,28]. The replisome-associated metalloprotease SPRTN (DVC1) plays a central role in DPC removal and repair, and mutations in the *SPRTN* gene are associated with a premature aging phenotype with early-onset hepatocellular carcinoma, known as Ruijs-Aalfs or SPARTAN syndrome [27–32].

Importantly, all of the structures mentioned in the preceding text can also result in topological constraints on DNA replication (Figure 1I), and torsional stress (e.g., from DNA supercoiling) can reduce fork speed and challenge fork stability [33–35]. We recently proposed that, analogous

DNA synthesis rate in response to DNA damage (e.g., in the presence of DNA DSBs).

Poly(ADP-ribose) polymerase (PARP) inhibition: drug-induced block of poly(ADP-ribose) synthesis by PARPs, entailing genotoxic PARP trapping.

Replication catastrophe: condition of massive replication stress-associated DNA damage during S phase; occurs after the excessive formation of ssDNA at stalled replication forks, which exhausts the ssDNA-protecting RPA pool.

Replication checkpoint: cell cycle checkpoint mechanisms to sense perturbations during DNA replication, such as replication fork stalling, and to coordinate fork remodeling and restart with origin firing and S-phase progression.

Replication origins: genomic loci from which DNA replication initiates. Origins are licensed for a new round of replication in late mitosis and early G1. Only approximately 10% of licensed origins fire in a temporally and spatially controlled manner during S-phase progression, while the rest remain silent.

Replisome: the multiprotein complex that, starting at replication origins, unwinds the parental DNA and synthesizes new DNA during the process of genome duplication.

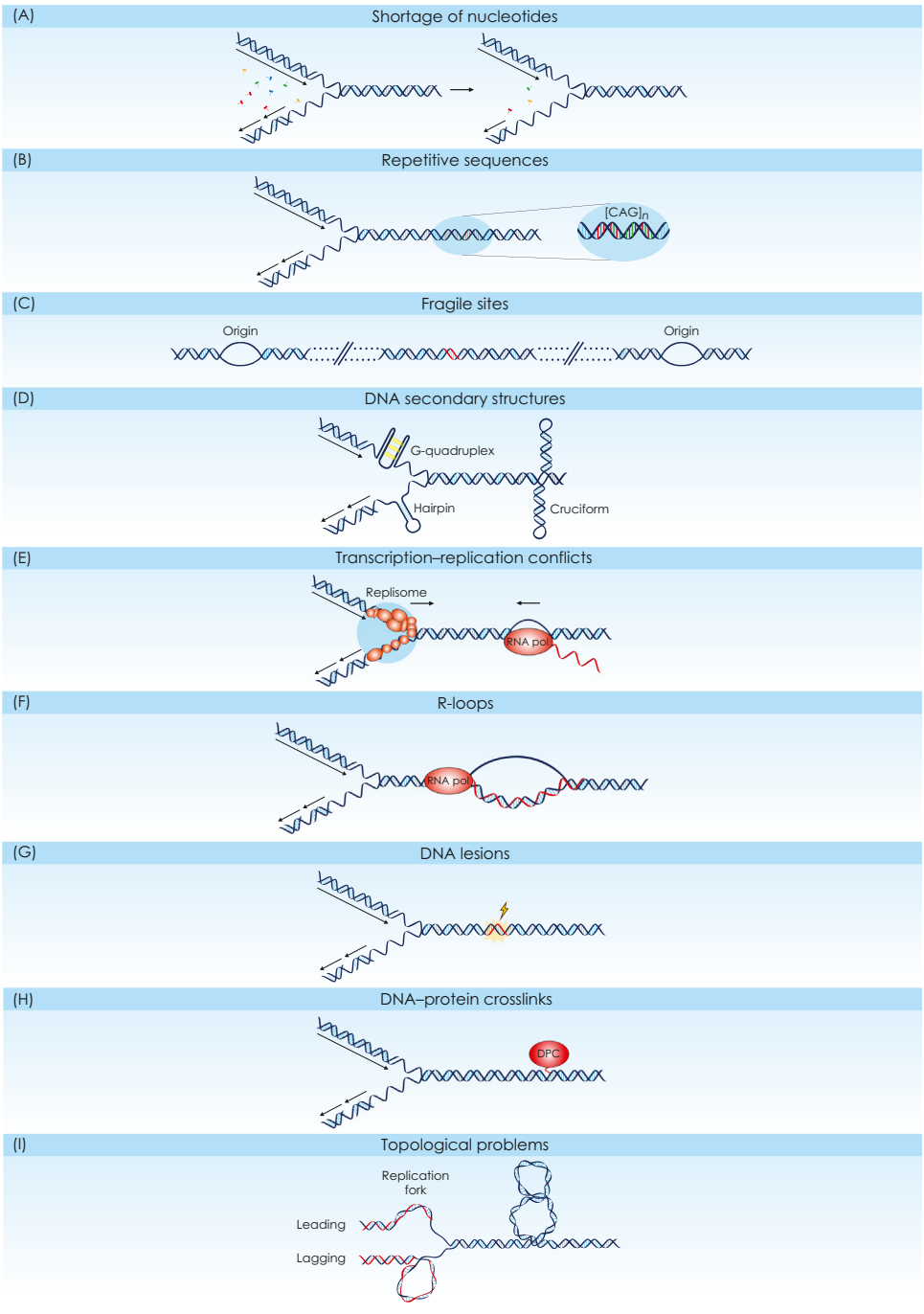


Figure 1. Endogenous Sources of Replication Stress. Replication stress can be caused by various impediments that interfere with the normal function of the replication machinery and impair replication fork progression. Sources of replication stress include (A) nucleotide shortage, (B) repetitive DNA sequences, (C) chromosomal fragile sites, (D) DNA secondary structures, (E) transcription–replication conflicts, (F) co-transcriptional R-loops, (G) replication fork-stalling DNA lesions, and (H) DNA–protein crosslinks, as well as (I) topological constraints that affect replication fork progression. Oncogene activation can exacerbate multiple causes of endogenous replication stress during cancer development.

to **gene gating** in yeast (i.e., the direct coupling of transcription to mRNA export by localizing transcription units close to the nuclear pore complex) [36], torsional stress arises when co-transcriptional mRNA cleavage is deregulated and replisomes encounter transcription units with unreleased nascent transcripts that localize close to the nuclear periphery [37]. This situation renders cells highly dependent on replication stress signaling, a condition that can be relieved by suppressed origin firing through cyclin-dependent kinase (CDK) inhibition [37]. Intriguingly, an analogous situation of gene gating occurs during oncogenic MYC expression to enable efficient transcription-coupled mRNA export [38]. It is thus tempting to speculate that, similar to exhausted pre-mRNA cleavage and impaired mRNA release from gene bodies [37], oncogene expression could also promote gene gating-associated replication stress and thereby enhance genome instability in cancer.

The Replication Checkpoint as a Rheostat for Replication Control

With more sensitive tools to investigate replication intermediates and endogenous genomic lesions, the border between normally occurring replication structures and DNA damage has started to blur. For instance, the **intra-S-phase checkpoint** has been defined as a mechanism to slow or stop replication in response to DNA double-strand breaks (DSBs) [39]. By now we know that replication forks undergo frequent fork reversal to form transient four-way junctions even in unperturbed conditions [6] and that the regressed arms of reversed replication forks resemble DSB ends, which use canonical DDR factors [40]. A clean distinction between DNA damage-induced and replication checkpoint signaling may therefore not always be possible. Moreover, as demonstrated recently for interstrand crosslinks (ICLs), not only replication forks that directly encounter lesions but also the majority of forks that are not directly affected by replication impediments slow in an ATR-dependent manner [41]. Thus, at least for certain replication impediments, local and global fork speed seem tightly connected by checkpoint kinase signaling.

ATR is the apical replication checkpoint kinase and together with its effector kinase CHK1 coordinates fork stabilization and fork speed, replication stress signaling, and origin firing. The latter is primarily achieved by CHK1-mediated phosphorylation and subsequent degradation of the CDC25A phosphatase, which reduces S-phase **CDK activity** by elevating inhibitory CDK phosphorylations. Under conditions of acute replication stress, ATR–CHK1 signaling globally inhibits the firing of late replication origins, thereby limiting the number of active replication factories, while at the same time allowing **dormant origins** in active factories to fire so that stalled replication forks nearby can be recovered [42,43]. Replication obstacles can also be overcome by fork remodeling, repriming, and post-replicative repair [10,44], and re-initiation of DNA synthesis past fork-stalling lesions, mediated by the primase-polymerase PrimPol, is emerging as an important mechanism to promote genome duplication under challenging conditions and to coordinate replication in space and time [45–47].

Overall, a picture of highly dynamic control of DNA replication and S-phase progression, buffered against the different sources of replication stress and fork stalling lesions, arises in which not only the type and severity of replication fork hindrance determine the outcome for genome integrity but also, importantly, how long replication problems and fork stalling lesions persist (Figure 2). Dynamic fork reversal is utilized when forks stall transiently, to remodel the fork structure into a four-way junction thereby stabilizing the fork and buying time for the removal of the fork slowing structure. Persistently stalled replication forks may use nucleolytic fork processing, repriming, template switching, and translesion synthesis as part of the DNA damage tolerance pathway. In more extreme cases, lasting replication blocks that cannot be bypassed can lead to fork collapse and recombination-mediated repair and fork restart [48]. As cells continue their progression through S phase, the time left to resolve replication stress-inducing lesions decreases and fewer options remain. A key function of cell cycle checkpoint control in this context is to ensure that the

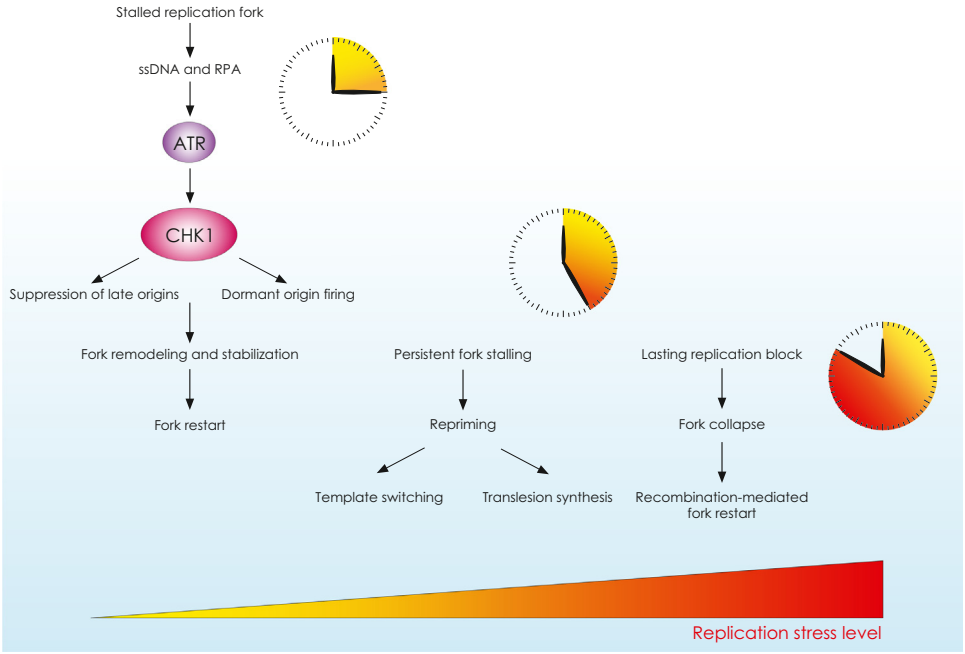


Figure 2. Fighting Against Time When Dealing with Replication Stress. Stalled replication forks trigger ataxia telangiectasia and Rad3-related protein kinase (ATR) activation, which, via checkpoint kinase 1 (CHK1) phosphorylation and its release from the chromatin, suppresses the firing of late origins, thus buying time to solve the problem. Dormant origins in active replication clusters are allowed to fire, however, and can rescue stalled replication forks under conditions of activated ATR–CHK1 signaling. Transiently stalled forks use fork reversal to remodel the fork structure into a four-way junction and thereby stabilize it. Persistently stalled replication forks may use nucleolytic fork processing, repriming, template switching, and translesion synthesis as part of the DNA damage tolerance pathway to overcome the problem. Lasting replication blocks can lead to fork collapse and recombination-mediated repair and fork restart. Unless replication is blocked completely (e.g., by global nucleotide depletion), S-phase progression does not necessarily stop in response to replication stress. Rather, depending on the type and severity of the lesion and its relative abundance, replication fork speed and origin firing are dynamically regulated while lesion removal, bypass, or damage repair occur simultaneously. As a consequence, unresolvable problems and persistently stalled replication forks build up an increasing replication stress level as cells continue to progress through S phase.

cellular buffering is not exhausted too easily [49]. The ssDNA-binding protein RPA plays a critical role in this buffering against endogenous and exogenous sources of replication stress, including conditions of elevated ssDNA formation when leading and lagging strand DNA synthesis are uncoupled [50]. Given the large excess of licensed replication origins, by tunable suppression of origin firing the checkpoint kinases ensure that ssDNA formation is normally restricted and that S-phase progression occurs within the limits of the replication capacity.

Intrinsic Replication Checkpoint Signaling

Early work investigated ATR and CHK1 functions mostly after induced DNA damage or severe replication stress. ATR and CHK1 are essential kinases, however, and both are required for normal S-phase progression (i.e., in absence of exogenous replication stress) [51–53]. The physiological importance of this signaling axis is underscored by mutations in *ATR* that cause Seckel syndrome, an autosomal recessive disorder characterized by intrauterine growth retardation, dwarfism, microcephaly, craniofacial abnormalities, and intellectual disability [54]. During unperturbed S-phase progression, ATR–CHK1 signaling continuously targets CDC25A for degradation [55–57]. This restricts CDK activity and suppresses origin firing [58–61], and basal ATR–CHK1 signaling inhibits premature activation of the mitotic kinase PLK1, thereby directly coupling DNA

replication to cell cycle progression and the timing of mitosis [62]. In other words, the replication process itself activates a built-in tunable cell cycle brake.

As continuous activation of CHK1 is linked to CHK1 stability [63], this built-in cell cycle brake is maintained as long as basal levels of CHK1 activity are being held up. Consistently, by the use of a live-cell CDK reporter, it was recently shown that intrinsic replication checkpoint signaling results in fluctuating changes in CDK2 activity during unperturbed S-phase progression, which constantly adjusts DNA synthesis rates [64]. Downstream of CDK activity, a FOXM1-dependent transcription program regulates the expression of mitotic genes, and the CDK1-mediated phosphorylation and activation of the transcription factor FOXM1 is antagonized by ATR–CHK1 signaling until replication is complete [65]. Intrinsic checkpoint signaling and endogenous replication stress thus link cell cycle progression to the completion of genome duplication. Besides physical obstacles to replication fork progression as triggers of intrinsic checkpoint signaling, limiting dNTP supply at the onset of S phase also causes checkpoint signaling to prevent overhasty replication [66]. Taken together, these findings suggest that intrinsic replication checkpoint signaling acts as a rheostat for replication and cell cycle control, and that, rather than resembling a checkpoint in the classical sense of the word, the ATR–CHK1 signaling axis can be viewed as a tunable brake, which protects proliferating cells from overheated DNA replication and from rushed or untimely cell cycle transitions.

The mechanisms that lead to the activation of ATR and CHK1 under unperturbed cell cycle progression remain incompletely understood. One of the players that seems to be involved is the ATR activator ETAA1 [63,65,67]. ETAA1 localizes at replication forks and binds to RPA, ATR, and ATRIP [68–70], suggesting that replication-associated ssDNA plays a role in intrinsic checkpoint control. Indeed, RPA-covered ssDNA stimulates ETAA1-dependent ATR activation *in vitro* [71]. ETAA1 also has mitotic functions [72,73], and additional sources of interphase ATR activation may exist. For instance, RNA polymerase II (RNAPII)-mediated transcription can induce ATR signaling, and inactivation of the RNAPII CTD phosphatase PNUTS-PP1 greatly enhances ATR-dependent phosphorylation [74]. Furthermore, it was recently shown that CHK1 activation under unperturbed conditions can occur through proteolytic cleavage of its C-terminal autoinhibitory region by the SPRTN protease. This releases signaling-competent CHK1 and reinforces SPRTN recruitment in a SPRTN–CHK1 cross-activation loop [75]. Downstream of CHK1 activation, its autophosphorylation status is important for intrinsic checkpoint maintenance. Mutants of CHK1, which are defective in autophosphorylation, show reduced stability due to enhanced proteasomal degradation, and small molecule inhibitors targeting replication checkpoint signaling disrupt intrinsic checkpoint maintenance [63]. While this sensitizes cells to acute replication blocks, in absence of exogenous replication stress it leads to accelerated cell cycle progression and replication-associated DNA lesions that are transmitted to daughter cells through cell division. Such heritable genomic lesions affect cell cycle transitions in the next cell generation, suggesting that intrinsic replication checkpoint signaling impacts cell cycle control far beyond S phase [76–78]. Signaling from replication remnants after cell division, resembling part of the replication stress history, is integrated by cells with mitogen signaling and mitogen history to control further proliferation [79–81]. Deregulated or impaired ATR–CHK1 signaling (e.g., by drug-induced kinase inhibition) may therefore amplify endogenous replication stress levels across multiple cell generations, with poorly understood implications for cell fate decisions and long-term cell survival.

CHK1 Regulation by Post-translational Modifications (PTMs) and Proteasomal Degradation

PTMs are critical for the regulation of ATR–CHK1 signaling, both under conditions of acute replication stress and during unperturbed DNA replication. CHK1 is activated by the upstream ATR kinase through phosphorylation at S317 and S345. The CHK1-interacting protein and coactivator

Claspin, which is also phosphorylated by ATR, supports this step by promoting CHK1 recruitment and facilitating its ATR-dependent phosphorylation [82]. Subsequently, CHK1 autophosphorylates its residue S296, which causes its release from chromatin and promotes checkpoint signaling [83,84]. Although several phosphatases are able to counteract CHK1 phosphorylation (Figure 3A) [85–88], autophosphorylated CHK1 can be detected during unperturbed S-phase progression and is linked to the maintenance of CHK1 stability [63,75]. Multiple ubiquitin ligases, including the SKP1-CUL1-F-box (SCF) complex, CUL4-DDB1, and HUWE1 [89–93] can target CHK1 for proteasomal degradation, whereas the deubiquitinases USP1, USP3, USP7, and Ataxin-3 [94–97] were shown to antagonize CHK1 ubiquitylation and degradation (Figure 3B).

Preventing unscheduled CHK1 inactivation and degradation during normal cell proliferation is critical for what we refer to as intrinsic checkpoint maintenance (Figure 4A). Under such conditions of mild endogenous replication stress, keeping CDK activity in check by a tunable CHK1-dependent cell cycle brake is important to maintain a sufficient buffering capacity and to avoid premature cell cycle transitions. After full checkpoint activation by high levels of replication stress or acute DNA damage, however, the main risk shifts towards excessive and prolonged checkpoint activation and an inability to resume cell cycle progression. Checkpoint signaling therefore needs to be terminated once replication stress is back under control and DNA lesions have been repaired. Such **checkpoint termination** is mediated by the inactivation and proteolytic degradation of CHK1 and of its positive regulator Claspin [98–101], indicating that, while CHK1 inactivation and degradation are dangerous for cells during normal replication and mild levels of replication stress, the same mechanisms are beneficial when cells need to overcome checkpoint activation and recover from acute checkpoint signaling (Figure 4B).

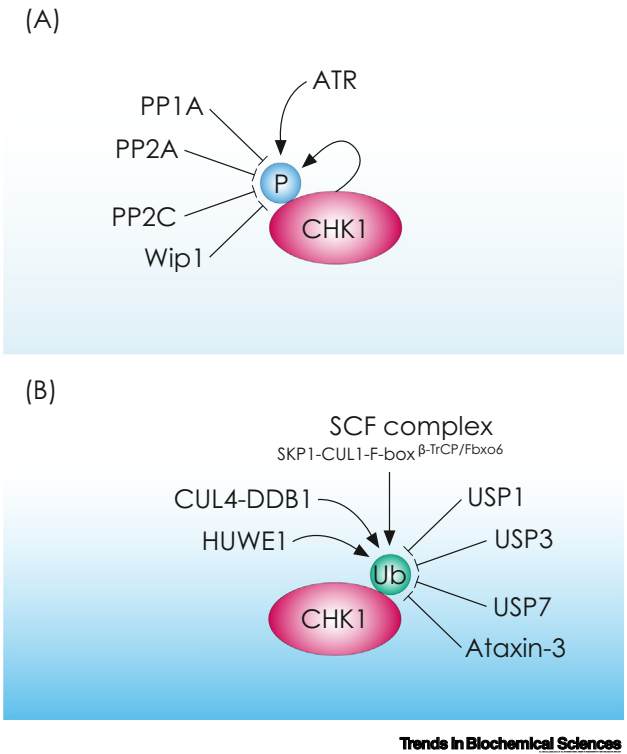


Figure 3. Phospho- and Ubiquitin-Mediated Regulation of Checkpoint Kinase 1 (CHK1) Activity and Stability. (A) CHK1 regulation by ataxia telangiectasia and Rad3-related protein kinase (ATR)-dependent phosphorylation (P) and subsequent automodification, antagonized by the activities of the indicated protein phosphatases. (B) CHK1 regulation by ubiquitylation (Ub) and proteasomal degradation, which are antagonized by the activities of deubiquitinases (DUBs).

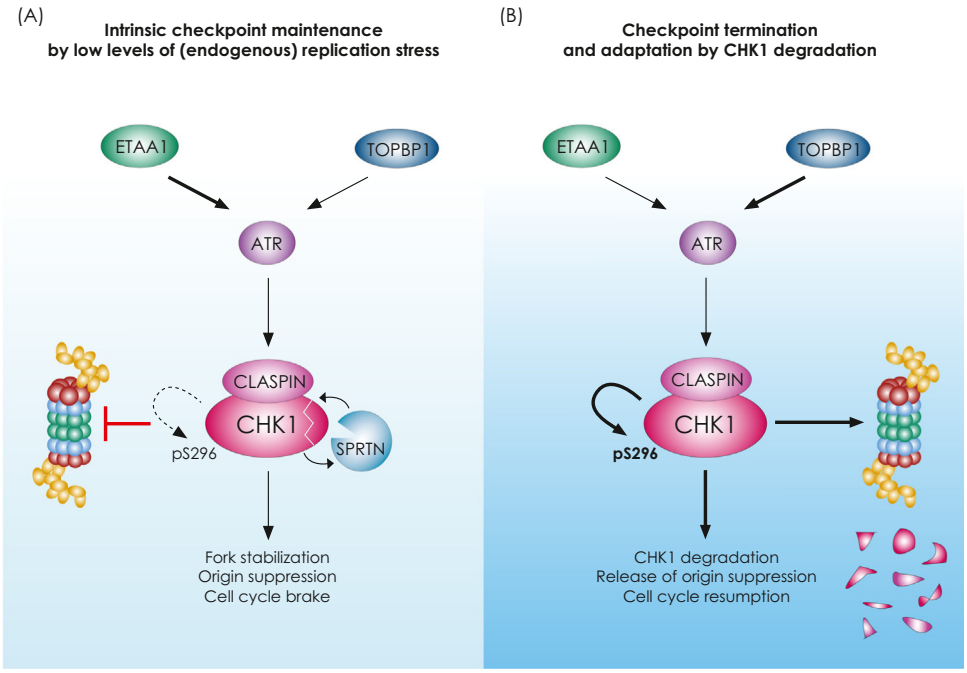


Figure 4. Checkpoint Kinase 1 (CHK1) Stability and Its Links to Checkpoint Activity. (A) Basal CHK1 activity during normal cell proliferation is needed to maintain CHK1 stability and thereby also maintain intrinsic checkpoint functions. While CHK1 stability is linked to its autophosphorylation status, basal CHK1 activity is also regulated by SPRTN-dependent removal of a C-terminal autoinhibitory region. (B) Persistent high levels of CHK1 activation can lead to proteasomal degradation of CHK1 and its activator Claspin, enabling checkpoint termination or checkpoint adaptation after acute or persistent genotoxic stress.

Finally, as opposed to checkpoint termination after resolved replication stress and repaired DNA damage, checkpoint adaptation as a response to persistent genotoxic stress has been described [102–104]. As the checkpoint loosens its grip, genomic lesions and incompletely replicated DNA are transmitted to G2/M for post-replicative repair and for DNA synthesis well beyond S phase. Such mechanisms, which can enlarge the proliferative capacity of cells under conditions that challenge genome integrity, and which provide opportunities for cellular evolution and pathological transformation, challenge the concept of stop and go cell cycle decisions and blur again the borders between conventionally defined cell cycle phases. Consistently, in cancer cells, segregation of pervasive, unrepaired lesions into subsequent cell generations shapes the evolution of cancer genomes and affects their adaptation to chemotherapy [105].

Targeting Replication Checkpoint Kinases for Cancer Therapy

Replication stress and checkpoint signaling can be seen as a continuum, from low and moderate endogenous replication stress during unperturbed DNA replication to exceedingly high replication stress levels after oncogene activation, hyperproliferation, or exposure to exogenous agents that induce severe replication problems and DNA damage. Within this continuum, cancer cells often show elevated replication stress and are closer to exhausting their replication capacity than non-cancer cells. Inhibiting ATR–CHK1 signaling, or blocking the related cell cycle kinase and CDK1 antagonist WEE1 (Box 1), has therefore become a promising avenue in targeted cancer therapy [54,106]. Several clinical trials have been or are currently testing ATR and CHK1 inhibitors and putative biomarkers to predict responses to checkpoint kinase inhibition are being evaluated [107]. Importantly, blocking the kinase activities of ATR, CHK1, or WEE1 synergizes not only with

Box 1. The WEE1 Kinase

The WEE1 kinase is a critical component of cell cycle regulation and G2/M checkpoint control. It exerts its function by restraining CDK activity via inhibitory phosphorylation, which in turn is counteracted by the CDC25A phosphatase. Similar to ATR and CHK1 inhibition, WEE1 deficiency leads to massive chromosome breakage during DNA replication [115]. WEE1 inhibition promotes unscheduled processing of replication intermediates in S phase, a process that is normally reserved for mitosis [116]. Additionally, WEE1 inhibition disturbs the temporal segregation between G1 and S phase, promoting premature S-phase entry and dormant origin firing [117]. Like ATR and CHK1, the WEE1 kinase is a promising target in cancer therapy, and WEE1 inhibition was recently shown to synergize with CHK1 inhibition in causing unscheduled origin firing and DNA damage in S phase [118]. Through untimely CDK activation, WEE1 inhibition also leads to degradation of the RNR subunit RRM2, thereby causing a shortage in dNTP supply. This is particularly important in H3K36me3-deficient cancers, where WEE1 inhibition-induced RRM2 destabilization synergizes with impaired *RRM2* transcription to cause excessive replication stress and cell death [119]. WEE1 inhibition may also be beneficial in conjunction with other genotoxic drugs such as PARP inhibitors, when applied either in combination or sequentially to reduce toxicity [120].

elevated endogenous replication stress in cancer cells, but also with genotoxic stress and DNA damage induced by orthogonal cancer therapies. For instance, ATR inhibition potentiates the irradiation-induced type I interferon response [108,109] and checkpoint kinase inhibition synergizes with dNTP depletion by gemcitabine, which enables the use of significantly lower drug concentrations, thereby alleviating common side effects such as myelosuppression [107].

Replication stress and replication stress-associated DNA lesions are also caused by **poly (ADP-ribose) polymerase (PARP) inhibition**, which is particularly detrimental for BRCA-deficient cancer cells [107,110]. Acquired PARP inhibitor resistance, a major clinical problem, can be overcome by inhibition of ATR or CHK1 [111,112]. At least in part, this could be due to cells entering mitosis prematurely with unresolved PARP inhibitor-induced lesions after inhibition of ATR–CHK1 signaling [110,113]. While this results in cytokinesis failure and mitotic catastrophe, it also leads to cells that re-enter the cell cycle, albeit with large amounts of genomic lesions inherited from the previous S phase [110,113]. Such heritable DNA damage, when allowed to enter the next S phase, will require very tight surveillance by the checkpoint machinery. If the replication checkpoint remains blocked, the likely outcome is excessive damage and **replication catastrophe**. If checkpoint inhibition is relieved too early, however, cancer cells may be able to cope with the extra load of DNA damage and, by reactivating cell cycle brakes and brake release mechanisms, complete DNA replication and resume proliferation – potentially with new mutations that may render them more aggressive. As promising as the therapeutic inhibition of replication checkpoint kinases is, in light of their essential functions and our currently limited understanding of the thresholds that define replication capacity under different conditions, a cautionary note on the therapeutic window and breadth of applicability seems expedient. The development of suitable biomarkers and combination therapies has already been initiated to address such concerns.

Concluding Remarks

While in previous years the focus was mostly on studying the hammer (i.e., the vigorous activation of cell cycle checkpoints to halt cell cycle progression in the face of acute and severe threats to genome stability), more sensitive methods and experimental readouts, including single-cell and single-molecule analyses, now allow more dedicated studies of the dance of cell cycle control (Figure 5). In particular, cell cycle-resolved single-cell analyses have the potential to capture the broad heterogeneity and the dynamics of cellular responses to endogenous replication stress and to correlate them with cell cycle position and checkpoint activation. The first examples have revealed that cell cycle boundaries and cell cycle checkpoints can be surprisingly loose, particularly in transformed cells [105,114]. When viewing the cell cycle as a continuum, with sequential yet often unsharp transitions, with a powerful engine fueled by CDK activity that drives

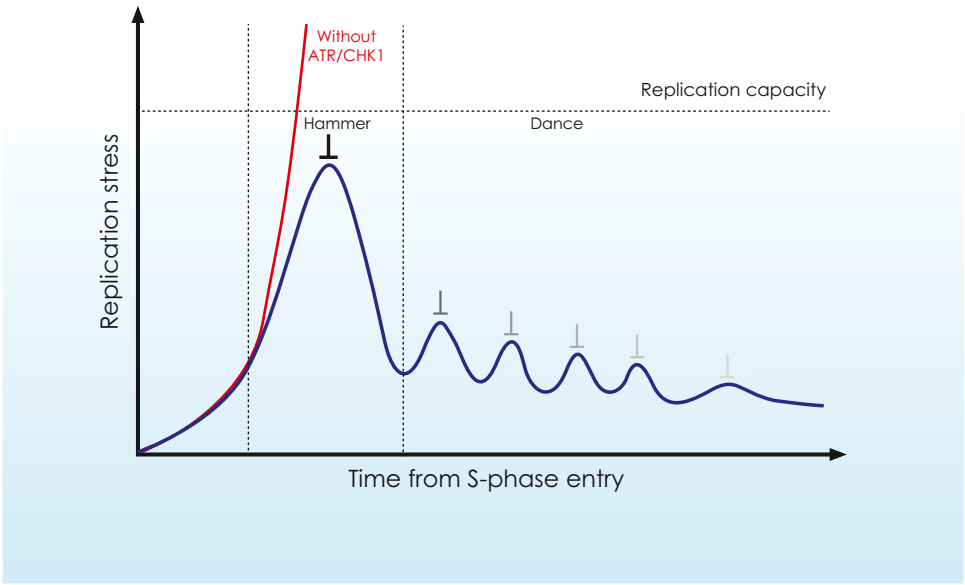
Outstanding Questions

How do multiple sources of ATR and CHK1 activation cooperate for intrinsic checkpoint signaling? What are the relative contributions of the different coactivators of ATR and CHK1? Which are, besides their previously characterized phosphorylation targets, the critical noncanonical downstream effectors of ATR and CHK1 activation?

Where in the cell does CHK1 degradation occur and how does it affect the firing of dormant versus late replication origins? Can CHK1 degradation occur on chromatin or is CHK1 release from chromatin a prerequisite for its degradation? Is the mechanism of ubiquitin-dependent CHK1 degradation conserved under conditions of impaired CHK1 activity and during checkpoint recovery?

Do the sources of intrinsic checkpoint activation and the cellular thresholds that determine brake and brake release versus full cell cycle arrest depend on the cell type and the epigenetic landscape? How does replication timing influence brake and brake release mechanisms?

How wide is the therapeutic window for inhibitors of essential cell cycle checkpoint kinases in cancer therapy? How much does it depend on the cell of origin? Which are the best biomarkers to predict therapy responses?



Trends in Biochemical Sciences

Figure 5. The Hammer and the Dance of Cell Cycle Regulation. In response to severe threats to genome integrity, when the capacity of the DNA replication and repair systems is in danger of becoming exhausted, strong cell cycle checkpoint activation can be seen as the hammer, which forcefully limits further damage and thereby helps to contain the distress. For instance, global inhibition of new origin firing by deoxyribonucleotide triphosphate (dNTP) depletion or slowdown of replicative polymerases prevents the formation of excessive amounts of unwound single-stranded DNA (ssDNA), at the cost of temporarily pausing the process of genome duplication. Such a stop decision, triggered by full ataxia telangiectasia and Rad3-related protein kinase (ATR) and checkpoint kinase 1 (CHK1) activation, is required to ensure that the replication capacity, including the capacity of fork protection mechanisms, is not exhausted and to thereby prevent catastrophic genome damage. When conditions are back under control, the capacity of the system is sufficiently large to cope with naturally occurring replication stress and spontaneously occurring DNA lesions in a more flexible manner, using brake and brake release mechanisms with fine-tuned regulation of the ATR and CHK1 checkpoint kinases and of cell cycle-driving cyclin–CDK activities. In keeping with 'the hammer and the dance' metaphor, normal cells take risks with genome-compromising potential seriously and exert tight control to minimize the damage, whereas cancer cells often ignore the risks and act sloppily in their response. Note that while in principle the hammer may be required at any time during S-phase progression to prevent exhaustion of the replication capacity, and may be preceded by a dance phase, the passive replication of excess origins during the course of genome duplication gradually lowers the risk of origin firing-associated DNA damage in both normal and cancer cells.

cell cycle progression forward and with tunable brakes and brake release mechanisms that dynamically control the ride, delaying certain tasks for the benefit of others and thereby ignoring conventionally defined cell cycle boundaries might simply be a way to balance resources and pay tribute to time being limited for cell cycle completion. Consistently, the replication checkpoint is likely to comprise various degrees of gradually tunable signaling strength, rather than resting either in an off or in an on state, and cells thus progress through S phase always with 'one foot on the brake'. However, several important points still need to be addressed in more detail; for instance, with regard to the sources of intrinsic checkpoint activation and the cellular thresholds of brake and brake release mechanisms (see Outstanding Questions). Only once a more quantitative, systematic, and comprehensive understanding is obtained, are targeted therapies using checkpoint kinase inhibitors, as standalone agents or in combination therapies, bound to intervene with the hammer and the dance of cell cycle control in a more defined and therefore more effective manner.

Acknowledgments

We apologize to all colleagues whose work could not be cited due to space limitations. We are grateful to Aleksandra Lezaja and Vincent Spegg for critical feedback on the manuscript and thank Biomed Graphics for professional assistance with figure preparation. M.A. acknowledges the Swiss National Science Foundation (SNSF) and the European Research Council (ERC) under the EU's Horizon 2020 research and innovation program for research support (SNSF grant PP00P3_179057 and ERC-2016-STG 714326).

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